Sofia Alexandra Camacho Pereira

Licenciada em Ciências de Engenharia do Ambiente

Analysis of the interaction of polycyclic aromatic compounds in a model organism: integration of genotoxic and histopathological effects

Dissertação para obtenção do Grau de Mestre em Engenharia do Ambiente

Perfil de Engenharia de Sistemas Ambientais

Orientador: Prof. Doutora Maria Helena Ferrão Ribeiro da Costa, Professora Associada com Agregação, Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa

Co-orientador: Doutor Pedro Manuel Broa Costa, Investigador Sénior do IMAR – Instituto do Mar

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À mamã, ao papá e à mana.

Agradecimentos

Não poderia deixar de agradecer a todas as pessoas que foram um importante apoio e ajuda constante ao longo do meu Mestrado, sem os quais não seria possível a realização desta dissertação e aos quais estarei eternamente grata.

À Professora Maria Helena Costa pela oportunidade de trabalhar neste projeto, pela orientação, e apoio, e por todo o conhecimento que me transmitiu ao longo do meu percurso académico.

Ao Pedro Costa pelo apoio constante no laboratório e orientação em toda a elaboração da tese, pela disponibilidade, por todos os conhecimentos que me transmitiu, por todos os conselhos, críticas e incentivo que me estimularam a trabalhar mais e não desistir, e por ter acreditado em mim.

À Marta Martins pelo apoio e disponibilidade em ajudar, pelos conselhos e incentivo ao longo do trabalho.

Aos meus colegas de laboratório Ana Patrícia, Carla, Cátia, João, Jorge e à Filipa por estarem sempre disponíveis para ajudar, pela partilha de conhecimentos e dicas importantes, pelas conversas e gargalhadas que me proporcionaram momentos de descontração.

Aos *migos* Cátia, Rita, Sílvia, Vanessa, Joana, José e Francisco pela amizade, pelo apoio, pela força nos momentos mais difíceis e também por todos os momentos de descontração e alegria.

Aos amigos "da Madeira" pela amizade e mensagens de apoio, e por me terem acompanhado nesta grande "viagem", mesmo estando longe.

Aos meus tios, tias, primos e primas e à família de coração, que mesmo longe estiveram sempre por perto. Especial obrigado à Joana e à Mariana pelos vossos conselhos, apoio e pelas viagens e aventuras que partilharam comigo.

À minha irmã por toda a força e amizade, pelos momentos de alegria e passeios ao fim da tarde que me fizeram esquecer os problemas e por me ter aturado nos momentos mais difíceis.

Por último, aos meus pais, um obrigado do tamanho do universo, por me terem permitido embarcar nesta "viagem", por terem acreditado em mim, por serem os principais responsáveis pela pessoa que sou hoje e pelo vosso apoio e amor incondicional que me permitiram ultrapassar todos os obstáculos e dificuldades.

Obrigado.

Abstract

Due to their toxicity, especially their carcinogenic potential, polycyclic aromatic hydrocarbons (PAHs) became priority pollutants in biomonitoring programmes and environmental policy, such as the European Water Framework Directive. The model substances tested in this study, namely benzo[b]fluoranthene (B[b]F), considered potentially carcinogenic to humans and an effector carcinogenic PAH to wildlife, and phenanthrene (Phe), deemed a non-carcinogenic PAH, are common PAHs in coastal waters, owning distinct properties reflected in different, albeit overlapping, mechanisms of toxicity. Still, as for similar PAHs, their interaction effects remain largely unknown. In order to study the genotoxic effects of caused by the interaction of carcinogenic and non-carcinogenic PAHs, and their relation to histopathological alterations, juvenile sea basses, *Dicentrarchus labrax*, a highly ecologically- and economically-relevant marine fish, were injected with different doses (5 and 10 $\mu g.g^{-1}$ fish ww) of the two PAHs, isolated or in mixture, and incubated for 48 h. Individuals injected with B[b]F and the PAH mixture exhibited higher clastogenic/aneugenic effects and DNA strand breakage in blood cells, determined through the erythrocytic nuclear abnormalities (ENA) and Comet assays, respectively. Also, hepatic histopathological alterations were found in all animals, especially those injected with B[b]F and the PAH mixture, relating especially to inflammation. Still, Phe also exhibited genotoxic effects in sea bass, especially in higher doses, revealing a very significant acute effect that was accordant with the Microtox test performed undergone in parallel. Overall, sea bass was sensitive to B[b]F (a higher molecular weight PAH), likely due to efficient bioactivation of the pollutant (yielding genotoxic metabolites and reactive oxygen species), when compared to Phe, the latter revealing a more significant acute effect. The results indicate no significant additive effect between the substances, under the current experimental conditions. The present study highlights the importance of understanding PAH interactions in aquatic organisms, since they are usually present in the aquatic environment in complex mixtures.

Key-words: PAH toxicity; Benzo[b]fluoranthene; Phenanthrene, Carcinogenic and non-carcinogenic PAHs, Histology, DNA strand breakage

Sumário

Os hidrocarbonetos aromáticos policíclicos (PAHs), devido à sua elevada hidrofobicidade e à sua toxicidade, especialmente o seu potencial carcinogénico, são considerados substâncias prioritárias em programas de biomonitorização e política ambiental, tais como a Diretiva-Quadro da Água. As substâncias modelo testadas neste estudo, benzo[b]fluoranteno (B[b]F), considerado potencialmente carcinogénico para os humanos, e fenantreno (Phe) considerado um PAH não carcinogénico, são compostos comuns em água costeiras, que possuem propriedades distintas refletidas em diferentes mecanismos de toxicidade. Ainda assim, como para PAHs semelhantes, os efeitos da sua interação são ainda desconhecidos. Com o objetivo de estudar os efeitos genotóxicos causados pela interação de PAHs carcinogénicos e não-carcinogénicos, e a sua relação com as alterações histopatológicas, robalos juvenis, *Dicentrarchus labrax*, foram injetados com diferentes doses (5 e 10 $\mu g.g^{-1}$ peso fresco) de dois PAHs, isolados e em mistura, e incubados por 48 h. Os indivíduos injetados com B[b]F e a mistura de PAHs apresentaram maiores efeitos clastogénicos/aneugénicos e quebras da cadeia de ADN nas células do sangue, determinados através do teste das anomalias nucleares eritrocíticas (ANE) e do ensaio *Comet*, respetivamente. Alterações histopatológicas hepáticas foram também encontradas em todos os animais, especialmente aqueles injetados com o B[b]F e a mistura de PAHs, relacionadas principalmente com resposta inflamatória. Ainda, Phe também causou efeitos genotóxicos no robalo, especialmente em doses elevadas, revelando um efeito agudo muito significativo, de acordo com o teste de Microtox realizado em paralelo. No geral, o robalo demonstrou sensibilidade ao B[b]F (PAH de maior peso molecular), provavelmente devido a bioactivação eficiente do poluente (produzindo metabolitos genotóxicos e espécies reativas de oxigénio), quando comparado com o Phe. Os resultados indicam que não existiu efeito aditivo significativo entre as duas substâncias, de acordo com as condições experimentais atuais. O presente estudo destaca a importância de compreender as interações dos PAHs em organismos aquáticos, uma vez que estão normalmente presentes em misturas complexas.

Palavras-chave: Toxicidade de PAHs; Benzo[b]fluoranteno, Fenantreno, PAHs carcinogénicos e não-carcinogénicos, Histologia, Quebra da cadeia de ADN

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Abbreviations

Ahr – Aryl Hydrocarbon Receptor
AO – Acridine Orange staining
ARNT – Aryl Hydrocarbon Nuclear Translocator
B[b]F – Benzo[b]fluoranthene
CYP – Cytochrome P450
DMSO – Dimethyl Sulfoxide
EDTA – Ethylenediamine Tetraacetic Acid
ENA – Erythrocytic Nuclear Abnormalities
EU – European Union
H&E – Hematoxylin and Eosin stain
IARC – International Agency for Research on Cancer
LMPA – Low Melting-Point Agarose
MFO – Mixed-Function Oxygenases
MMC – Melanomacrophage Centers
MSFD – Marine Strategy Framework Directive
NMPA – Normal Melting-Point Agarose
PAH – polycyclic aromatic hydrocarbons
PBS – Phosphate-Buffered Saline
Phe – Phenanthrene
ROS – Reactive Oxygen Species
SCGE – Single-Cell Gel Electrophoresis
SDI – Strategic Diagnostics Inc.
TAE – Tris-Acetate-EDTA
USEPA – United States Environmental Protection Agency
WFD – Water Framework Directive
XRE – Xenobiotic Response Element

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1. Introduction

Aquatic ecosystems are constantly subjected to a cocktail of toxic chemicals that result from the combination of different anthropogenic pressures, such as industry, agriculture, transport and urbanism. As a consequence, water pollution became a serious threat to human populations and the biota that may be exposed to contaminants, e. g. through water and food, besides severe socio--economical impacts. In the developed world, legislation has been issue to try to meet the growing threat of water pollution. On October 2000, the European Union (EU) adopted the EU Water Framework Directive (WFD, Directive 2000/60/EC), updated in 2008, to which is added the Marine Strategy Framework Directive (MSFD, Directive 2008/56/EC), aiming at establishing the legislative grounds for the safeguard of inland and coastal aquatic ecosystems and water quality. These directives set guidelines and standards to minimize and monitor the adverse impacts of human activities on aquatic ecosystems. The WFD is complemented by the List of Priority Substances (Annex X of the WFD) within which eight Polycyclic Aromatic Hydrocarbons (PAHs) are included: naphatalene, anthracene, fluoranthene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, benzo[ghi]perylene and indenol[1,2,3-*cd*]pyrene.

Polycyclic aromatic hydrocarbons are a particular class of organic pollutants, widely dispersed in the aquatic environment and constituted by hundreds of individual substances (see Douben, 2003). Environmental toxicology of PAHs and monitoring of aquatic environments can be changeling because they are usually present in the environment as complex mixtures and not as single chemicals. The compounds are typically formed by the combustion of organic matter, such as fossil fuels and forest fires (pyrogenic sources), or by geological process (petrogenic sources), such as petroleum (see Douben, 2003). These compounds consist essentially of carbon and hydrogen and have two or more fused aromatic rings. Variations in molecular weight will result in different physical and chemical characteristics (such as solubility in water), which determine its movement and environmental fate (see Douben, 2003, for review). The PAHs with lower-molecular-weight are usually associated with the two or three aromatic rings, whereas higher-molecular-PAHs are composed with four or more rings. Two or three PAH-ringed tend to diffuse more readily in water, whereas larger PAHs, due to their high hydrophobicity, tend to be trapped and stored in aquatic sediments (Douben, 2003).

Polycyclic aromatic hydrocarbons are considered priority in biomonitoring programmes, due to their high toxicity, with particular respect to their potential mutagenic and carcinogenic effects. The International Agency for Research on Cancer (IARC) classified PAHs as non-carcinogenic,

potentially carcinogenic and carcinogenic to humans. However, the differences between the toxicological mechanisms of either type (carcinogenic and non-carcinogenic) are not yet fully understood and, moreover, their interaction effects. These toxicants owe their toxicity to their ability of being metabolized by CYP (cytochrome P450) mixed-function oxygenases (MFOs) during phase I of detoxification (a process termed bioactivation), which results in highly reactive and toxic PAH metabolites and oxygen radicals as a secondary metabolites. In fish as for other vertebrates, the liver is the main organ involved in accumulation and detoxification of xenobiotics like PAHs and the majority of pollutants (see Tuvikene, 1995, for a review on PAH metabolism). Depending on parent compound and CYP isozyme to which it preferentially binds to, some PAH metabolites are highly genotoxic, eliciting strand breakage and/or adducts. The lesions, depending on whether they may be repaired or not, may result in mutations, potentially leading to carcinogenic effects (e.g. Aas *et al.*, 2000; Shimada and Fujii-Kuriyama, 2004; Vijayalakshmi and Suresh, 2008). In addition, ROS produced during bioactivation affects almost every aspect of intracellular metabolism, causing, inclusively direct DNA base oxidation (Livingstone, 2001). Although, the role of CYP P450 monooxygenase system in PAHs metabolic activation is long known to be linked with genotoxicity and carcinogenicity of these compounds (e.g. Conney, 1982), the division between "carcinogenic" and "non-carcinogenic" PAHs is mostly based on information retrieved from studies with mammals and applies especially to human risk, even though the mechanisms of PAH bioactivation and mutagenesis are ubiquitous at least between all vertebrates. Furthermore, studies focusing on the interaction effects between these two types of toxicants are essentially absent even though PAH bioactivation and detoxification processes are complex, interlinked and known to be affected by many factors, including the co-effects of other toxicants (see e.g. Whyte *et al*., 2000).

At the molecular level, PAHs metabolites may bind covalently to a nucleobase, forming adducts, and as consequence produce a modification of the DNA molecule (e.g. Gravato and Santos, 2002, 2003; Aas *et al.*, 2000). Whole-chromosome breakage can also occur by the action of PAHs, and result in an increase in frequency of structural (clastogenic alterations) and numerical (aneugenic alterations) aberrations in chromosomes (see e.g. Costa *et al.*, 2008, 2011a). So, genotoxicity assessment has been integrated in biomonitoring programs and is frequently applied in a wide range of organisms. Various techniques has been employed to determine the genotoxicity of PAHs in fish, including the analysis of erythrocytic nuclear abnormalities (ENA) assay (a variation of the micronucleus test that takes advantage of the nucleated red blood cells of fish) and the single-cell gel electrophoresis (SCGE) or Comet assay, as it is known. These two assays allow quantitating DNA damage at the level of nucleotide chains (DNA strand breakage) and at

the chromosomal level, respectively. The ENA assay are recorded the frequency of cells exhibiting nuclear alterations, such as micronuclei, fragmentations and budding (Costa and Costa, 2007). The alkaline version of the Comet assay determined DNA chain fragmentation resulting from the combination of single- and double-strand breaks, alkali labile sites (base or phosphate alkylation decomposed to form strand breaks, dependending on pH and time of alkaline treatment) and PAH-DNA adducts, that eventually break during electrophoresis (Singh *et al*., 1988). The co--employment of these two techniques has already proposed before for a more efficient assessment of genotoxicity in fish (e.g. Costa *et al*., 2008, 2011a; Neuparth *et al*., 2009).

Histopathological analyses in fish have become an important tool in aquatic biomonitoring programs and are acknowledged to be efficient and sensitive tools, since histopathological alterations tend to reflect the true state of health of the organism (e.g. Stentiford *et al*., 2003; Costa *et al*., 2009, 2011b, 2013). However, there is a lack of knowledge on the specificity of lesions and alterations on anatomy and pathology of fish and other aquatic organisms, being far better described for mammals (see Au, 2004; Costa *et al*., 2009 and references therein). Histopathological alterations can be determined qualitatively (description of histological lesions and alterations), semi-quantitatively or quantitatively (quantification of histological lesions and alterations by direct measurements to provide numerical data). However, it is the first case that produces the bulk of histological references on fish toxicological studies, especially those integrating multiple endpoints. In fact, although semi-quantitative and quantitative histopathological analyses may allow establishing cause-effect relationships statistically, it is not always possible or logistically feasible to undertake direct measurements on histopathological traits.

Dicentrarchus labrax (Moronidae, Perciformes), better known as the European sea bass, is a demersal teleost fish species that inhabits estuaries, lagoons, rivers and coastal waters and their geographical distribution extends over all waters around Europe, from the coastal waters of the Atlantic Ocean, to the Mediterranean Sea and the Black Sea, including Portuguese coastal ecosystems. They can be found in coastal waters and estuaries in summer, and in deep colder waters, in the northern range, during winter. As juveniles, sea basses form schools, unlike the adults who are more solitary animals. The species feeds principally on shrimps, mollusks and also fishes. *Dicentrarchus labrax*, as a representative estuarine species, has a high commercial value and economic importance for fish farming and aquaculture. In addition to its high ecological relevance, the sea bass hold high sensitivity to pollution and is known to possess efficient biotransformation abilities for PAHs, therefore being considered an adequate model organism for

ecotoxicological research (see for instance Gravato *et al*., 2000; Gravato and Santos, 2002, 2003). However, only a few studies concerning to the biotransformation system of xenobiotics of this species have been performed, mostly related to exposures of individual substances. The effects of mixed PAHs have yet to be surveyed in this species in order to disclose its full potential as a candidate sentinel organism.

2. Objectives

The main objective of this thesis is to evaluate the interaction effects, and at least part of their underlying mechanisms, between carcinogenic and non-carcinogenic PAHs in an ecologically relevant model teleost, the sea bass *Dicentrarchus labrax*, with particular respect to genotoxicity, for being the basis of potential mutagenic and carcinogenic effects. Two PAHs were surveyed, as model toxicants: phenanthrene (Phe), classified as non-carcinogenic to humans, and benzo[b]fluoranthene (B[b]F), considered potentially carcinogenic to humans and effectively carcinogenic to fish (IARC, 1983). Phenanthrene is a low molecular weight PAH is composed of three fused benzene rings. Although is not considered as mutagenic or carcinogenic (IARC, 1983), its toxic effects to aquatic organisms, such as ROS production, has been reported by various authors (Martins *et al*., 2013; Yin *et al*., 2007; Sun *et al*., 2006; Mu *et al*., 2012; Hannam *et al*., 2010; Xu *et al*., 2009; Correia *et al*., 2007) and it is included in the 16 USEPA priority PAHs (USEPA, 2008). Benzo[b]fluoranthene, which toxicological data is still scarcer, is a high molecular weight PAH, composed of five aromatic rings. B[b]F is considered as a high-risk environmental pollutant and its genotoxic effects to marine aquatic organisms has already been reported (Martins *et al*., 2013; Kerambrun *et al*., 2012). The choice of these two substances as models also related to their ubiquitous presence in marine environments, especially sediments, which act as the major storage of PAHs in coastal environments (see Martins *et al*., 2013).

The layout of the thesis is present at Figure 2.1. Specifically, it is aimed at:

- Comparing the genotoxic and histopathological effects of carcinogenic (B[b]F) and non-carcinogenic (Phe) PAH in a marine fish known to be able to bioactivate PAHs.
- Determining the type of DNA lesions (i.e. strand breakage or clastogenesis/aneugenesis) triggered preferentially by either compound.
- Identifying histological lesions and alterations in the liver (due to its role in toxicant metabolism) and the spleen (a hematopoietic organ) of *Dicentrarchus labrax*.
- Determining the potential interaction effects between the two substances *in vivo* through bioassays with isolated and mixed PAHs and several doses.
- To distinguish between chronic and acute toxic effects *in vivo* through genotoxic and histopathological endpoints, and integrate the information with the Microtox test to determine acute toxicity.

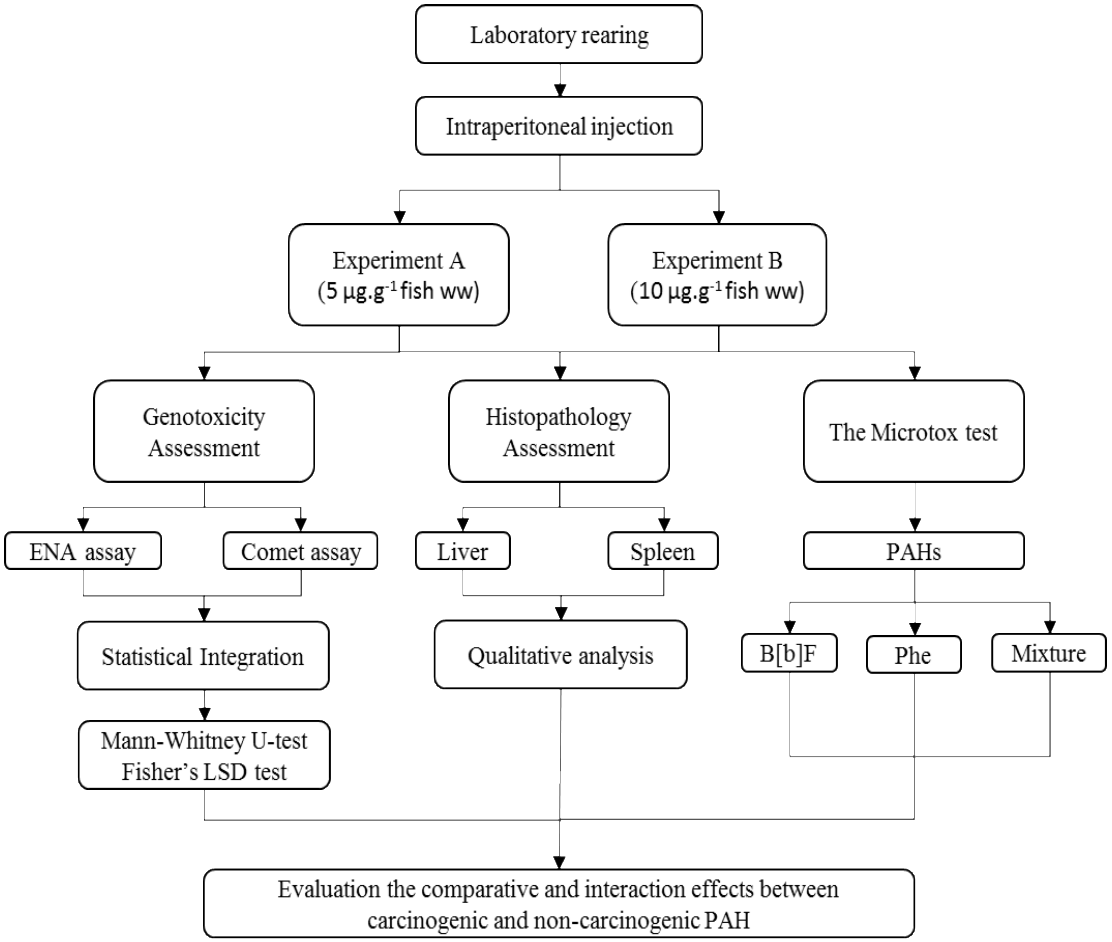

Figure 2.1. Thesis layout. ENA - erythrocytic nuclear abnormilites. B[b]F - benzo[b]fluoranthene. Phe - phenanthrene.

3. Material and Methods

3.1. Experimental procedure

Juvenile sea basses were injected intraperitoneally with two different doses of PAHs, 5 µg/g fish ww and 10 µg/g fish ww, prepared as solution in dimethylsulfoxide (DMSO), termed experiments A and B, respectively. For each experiment, A ("low dose") and B ("high dose"), animals (standard length = 76.62 ± 5.18 mm; total wet weight = 9.54 ± 1.93 g) were injected with either compound, isolated or in mixture, and divided by four treatments groups. These treatments were control (DMSO only), benzo[b]fluoranthene, phenanthrene and mixture, identified by C, B[b]F, Phe and M, respectively. Animals were injected with 100 µL of DMSO. The parental solutions contained 0.5 or 1 µg PAH/ µL DMSO for experiment A and B, respectively. After administration of the treatments, fish were maintained in 12 L-capacity white polyvinyl tanks with blunt edges to which 10 L of clean water was added. The bioassay was performed under temperature = 18 ± 1 °C, salinity = 32 ± 1g L^{-1}, pH = 7.9 ± 0.2, dissolved O_2 ranged between 92 and 95% and total ammonia was less than 0.05 mg L^{-1}. Photoperiod was set at 16:8 h light:dark. The tanks were continuously aerated and water parameters were monitored weekly, where 25% of total water volume was changed in order to maintain the bioassay conditions and ensuring minimal stress to the tested fishes. Sea basses were fed once a day with M2 grade commercial fish pellets (AQUASOJA, Ovar, Portugal). At the end of the incubation period (48 h) at least twelve individuals (six from each experiment A and B) per treatment, were sacrificed for genotoxic assessment and histological analyses.

3.2. Determination of genotoxicity

Genotoxic effects were assessed by the erythrocytic nuclear abnormalities (ENA) assay and the alkaline version of single-cell gel electrophoresis (Comet) assay in fish peripheral blood. Blood was collected just above the lateral line system with a syringe previously washed with 0.1 M EDTA anti-coagulant. To ensure maximum cell viability and minimum accessory damage analysis was run immediately after blood collection. Blood aliquots were smeared on glass microscopy slides for ENA analysis and diluted (1:200) in cold 50 mM PBS (phosphate-buffered saline) with 0.7% NaCl, pH 7.4 for the Comet assay.

ENA analysis was performed, as described by Costa and Costa (2007), on methanol-fixed blood smears (15 min), stained with 0.1 g L^{-1} acridine orange (AO) fluorochrome for 30 min (Sigma;

maximum absorbance at 488 nm). Slides were afterwards mounted with DPX (from BDH). Approximately 1000 intact mature erythrocytes were scored per slide in order to determine the percentage of cells with nuclear abnormalities. The ENA considered were: micronuclei, nuclear buds, polynucleated cells and fragmenting nuclei. Results are expressed as the percentage of mature erythrocytes showing nuclear abnormalities.

The Comet assay was prepared according to the protocol described by Costa *et al.* (2008), adapted from Singh *et al.* (1988). Blood cell suspensions (10 µL) were diluted in 180 µL of liquid (35-40ºC) 1% (m/v) low melting-point agarose (LMPA; Sigma) in PBS and placed (2 × 75 µL) on slides pre-coated with dried (for at least) 1% (w/v) normal melting-point agarose (NMPA) in TAE (Tris-Acetate-EDTA) buffer. After LMPA solidification (15 min, 4 ºC, in dark) slides were immersed for 1 h, in the dark, at 4 ºC, in lysis solution (2.64% NaCl w/v, 3.72% EDTA w/v and 5mM Tris), to which was added 10% (v/v) DMSO and 1% (v/v) Triton-X 100 just before use. To allow DNA unwinding and to enhance alkali-labile sites expression, slides were immersed in cold (4 ºC) alkaline electrophoresis solution (0.1 µM EDTA, 0.3 M NaOH; pH 13) for 40 min. Electrophoresis was performed at 25 V using a Sub-Cell model 96 apparatus (Bio-Rad), at 4 ºC for 30 min. Afterwards, slides were neutralized in 0.1 M Tris-HCl buffer (pH 7.5) for 15 min. All steps were executed under dim light and in cold (4 ºC) to prevent accessory DNA damage. The room temperature was controlled (≈ 20 ºC) to avoid agarose lifting from slides as well as to minimize procedural damage to DNA. One-hundred cells were scored per slide, after staining with 0.02 $mg.mL^{-1}$ ethidium bromide.

The percentage DNA in the tail, tail moment and Olive tail moment were considered as Comet metrics (Lee and Steinert, 2003). Tail parameters were calculated automatically using the software CometScore 1.5 (TriTek Corp., Summerduck, USA). The Olive tail moment was calculated by multiplication of percentage of DNA in tail and the length between the center of the head and the tail of the comet (Olive *et al*., 1990). However, the tail moment, also expressed in some studies as the extent tail moment (Lee and Steinert, 2003; Lee *et al*., 2004), was defined by percentage of DNA in tail multiplied by the tail length (length of DNA migration). Results are expressed in average percentage of DNA strand breakage per individual.

For both analyses was used a DMLB fluorescence microscope fitted with an EL6000 light source for mercury short-arc reflector lamps, equipped with an I3 filter, used for acridine-orange staining (AO), and an N2.1 filter, used for ethidium-bromide staining. All equipment was obtained from Leica Microsystems.

3.3. Histopathological procedure

All surviving animals, from each experiment A and B, were sacrificed by cervical sectioning and dissected immediately. Portions of liver and spleen were prepared for histological analyses essentially according to Martoja and Martoja-Pierson (1967). Liver was chosen as target organ due to its role in toxicant metabolism and spleen as a hematopoietic organ to evaluate the toxic effects of the PAHs in the immune system of the fish. Liver and spleen samples were immersed in Bouin-Hollande's solution (10% v/v formaldehyde and 7% v/v acetic acid to which picric acid was added till saturation) and fixed for 36 h, at 4 ºC. After fixation, the Bouin-fixed samples were washed in distilled water to remove excess picric and dehydrated in a progressive series of ethanol dilutions. Afterwards, samples were washed in xylene for intermediate impregnation and embedded in paraffin.

Sections of liver and spleen (5 µm thickness) were cut with a Jung RM2035 model rotary microtome (Leica Microsystems). A minimum of eight serial sections, for at least two slides per individual and per organ, were obtained and allowed to dry for 24 h at room temperature. Afterwards, liver and spleen samples were deparaffinated in of xylene, rehydrated to water following a regressive series of ethanol and stained with haematoxylin, for 2 minutes, being blued in tap water. Counterstaining was achieved with alcoholic eosin Y, for 1.5 minutes (H&E staining). Afterwards, samples were dehydrated in a progressive series of ethanol (70, 95 and 100%), cleared with xylene, allowed to dry completely at room temperature and mounted with DPX resin (from BDH).

The aforementioned microscopy apparatus was used for histological analyses.

3.4. Microtox test procedure

The acute toxicity of the compounds was evaluated by the Microtox test, through determining the luminescence inhibition of the marine bacterium *Vibrio fischeri*, following manufacturer instructions (Modern Water, Guilford, UK). In brief: after re-hydration of freeze-dried bacteria, the reconstituted *V. fischeri* were exposed to PAHs, isolated or in mixture, from each experiment A and B, and tested in duplicate, for 25 min. The tested PAH solutions were those used for injecting fish (see above). The DMSO proportion was 5% of the total volume in the test tubes.

Test reagent, diluent, osmotic adjusting solution, reconstitution solution and all the other materials necessary for analysis were supplied by Strategic Diagnostics Inc. (SDI, USA). The light emission was measured using the Microtox 500 analyzer (SDI, USA). Acute toxicity determination was performed accordingly to the basic test protocol, using the MicrotoxOmni software (SDI, USA). Results are expressed in percentage of effect (inhibition of luminescence) to *Vibrio fischeri*.

3.5. Statistical Analysis

After invalidation of the normality and the homogeneity of variances assumption for parametric analyses (determined through Kolmogoroff-Smirmoff and Levene's tests, respectively), ENA assay data were analyzed by the non-parametric Mann-Whitney *U* test for pairwise comparisons between treatments. Comet assay data were validated for the assumption for parametric analyses, and thus analyzed using one-way ANOVA followed by Fisher's LSD test as post-hoc procedure. A significance level α was set at 0.05 for all analyses. All the statistical tests were performed using the software Statistica 8.0 (Statsoft, USA).

4. Results

4.1. Mortality

Overall mortality was different between A and B experiments and, additionally, a distinct pattern was observed between the four treatments (C, B[b]F, Phe and M), as presented in Table 4.1.

Table 4.1. Mortality (given as number of casualties per six biological replicates) observed for the treatments C (control treatment), B[b]F (the potentially carcinogenic PAH), Phe (the non-carcinogenic PAH) and M (the combination of the two compounds). Results are shown for each experiment: A (lower dose, 5 $\mu g.g^{-1}$ fish ww) and B (higher dose, 10 $\mu g.g^{-1}$ fish ww).

	C	**B[b]F**	**Phe**	**M**
A	1 (17%)	1 (17%)	2 (33%)	0 (0%)
B	2 (33%)	1 (17%)	3 (50%)	4 (67%)

In experiment A (lower dose, 5 $\mu g.g^{-1}$ fish ww), exposure to Phe yielded the highest level of mortality (2 out of 6 individuals), followed by treatment C (control) and exposure to B[b]F, whereas the mixture treatment caused no mortality. Conversely, experiment B (higher dose, 10 $\mu g.g^{-1}$ fish ww) caused the highest level of mortality: 67% for treatment M followed by Phe and C, attaining 50% and 33%, respectively.

4.2. Genotoxicity Assessment

4.2.1. Erythrocytic nuclear abnormalities analysis

Nuclear buds and fragmenting nuclei represented the majority of nuclear alterations observed in mature erythrocytes (Figure 4.1 d, e, f and g). Nuclei that simultaneously presented various types of abnormalities were also recurrently found, like nuclear buds combined with fragmentation (Figure 4.1 f). It is possible that nuclear buds are the first stage of a micronuclei formation and fragmenting nuclei the origin of polynucleated cells (Costa and Costa, 2007). Sea basses erythrocytes also exhibited immature erythrocytes (Figure 4.1 a), micronuclei (Figure 4.1 i) and defence cells, mostly lymphocytes (Figure 4.1 j). Immature erythrocytes were distinguished from mature erythrocytes by presenting a more spherical shape and a larger and rounder nucleus. Micronuclei were identified by being completely separated from the main nucleus, with a diameter less than one-third of the main nuclei (Figure 4.1 i), following the criteria of Costa and

Costa (2007). Overall, the observed nuclear alterations are in accordance with previously studies for analysis of ENA in fish erythrocytes based on acridine orange staining (AO) (Costa and Costa, 2007).

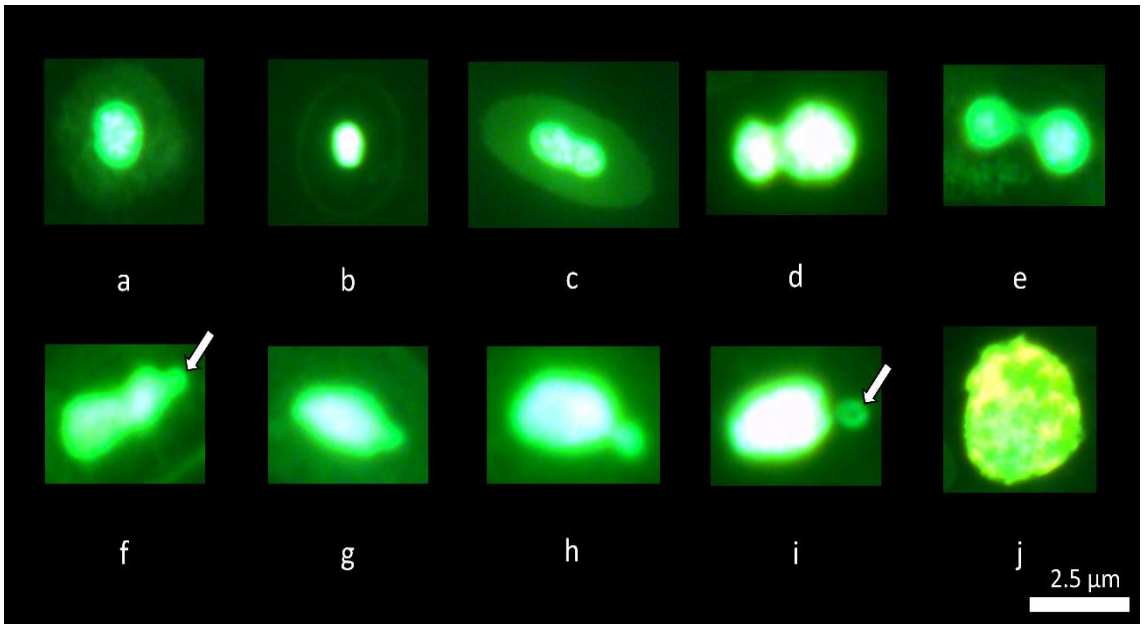

Figure 4.1. Normal nuclei and common ENA observed in erythrocytes of Dicentrarchus labrax. (a) normal immature erythrocyte; (b) normal mature erythrocyte; (c-i) mature erythrocytes exhibiting nuclear abnormalities: (c) lobed nucleus, (d-e) stages of fragmenting nuclei to form a binucleated cell, (f) lobed nucleus with nuclear bud (arrow), (g-i) different stages of nuclear bud until the formation of a fully individualized micronucleus (arrow); (j) lymphocyte.

Analysis of ENA frequencies showed a distinct pattern between experiments A and B, as presented in Figure 4.2. For experiment A, the overall differences were moderate, with the percentage of cells exhibiting ENA was similar between treatments C and Phe (%ENA > 11%) and between B[b]F and M (%ENA > 16%). Animals injected with PAH mixture and benzo[b]fluoranthene isolated exhibited moderate frequencies of mature erythrocytes with evidence of clastogenic and aneugenic alterations (Figure 4.2 A). However, in this experiment, the lowest induction of ENA was observed in fish treated with phenanthrene (Figure 4.2 A). Fish erythrocytes from the M treatment showed a significant increase of ENA frequency compared to the Phe treatment (Mann-Whitney *U* test, $p < 0.05$).

On the other hand, animals from experiment B exhibited a higher percentage of red cells presenting nuclear abnormalities for treatment B[b]F (25.11% of ENA) (Figure 4.2 B). Fish injected with benzo[b]fluoranthene induced the frequency of cells exhibiting ENA up to two-fold when compared to the control treatment (12.05% of cells with ENA) (Mann-Whitney *U* test, p <

0.05). The lowest induction of ENA was observed in fish injected with DMSO only (the control treatment) and the PAH mixture (Figure 4.2 B). However, animals injected with phenanthrene showed moderate frequencies of mature erythrocytes exhibiting nuclear abnormalities (Figure 4.2 B).

Overall, experiment B was the responsible for the highest induction of ENA, led by exposure to benzo[b]fluoranthene isolated (Figure 4.2 B). Still, both experiments yielded similar values for the control treatment (injection with DMSO only), revealing unaltered solvent effect between experiments (Figure 4.2).

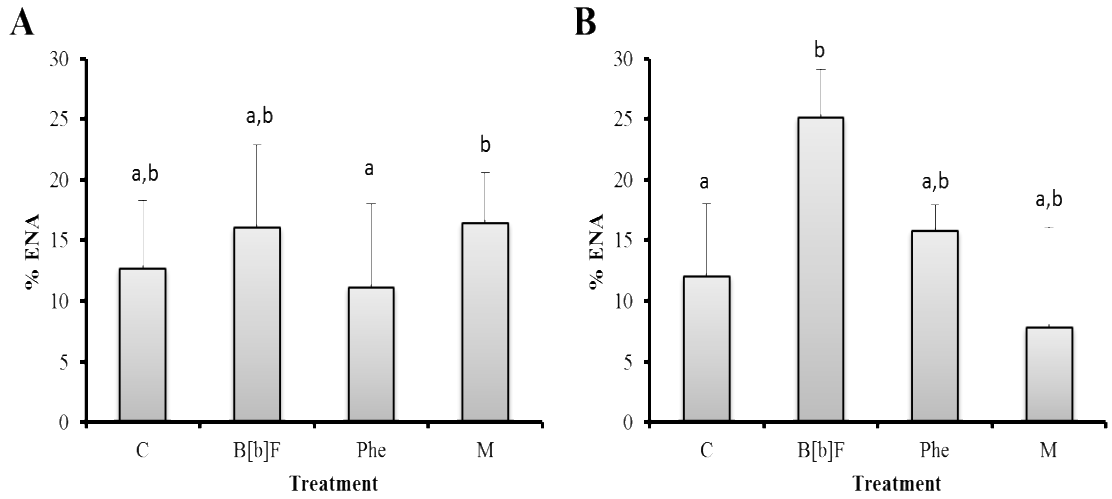

Figure 4.2. Mean percentage of cells showing ENA (1000 mature erythrocytes counted per individual). (A) Experiment with the lower dose (5 µg.g^{-1} fish ww). (B) Experiment with the higher dose (10 µg.g^{-1} fish ww). Error bars represent 95% confidence intervals. Different letters indicate significant differences (Mann-Whitney *U* test, $p < 0.05$). C - control treatment. B[b]F - treatment with benzo[b]fluoranthene (higher molecular weight PAH). Phe - treatment with phenanthrene (lower molecular weight PAH). M - treatment with the combination of the two PAH compounds.

4.2.2. The alkaline single-cell gel electrophoresis (Comet) assay

The Comet assay yielded distinct patterns of total DNA strand breakage (inferred from the % of DNA in tail) between experiments A and B (Figure 4.3). Overall, these patterns were consistent between metrics, i.e., %DNA in tail, tail moment and Olive moment (Figure 4.4).

Animals from experiment A showed in general moderate differences, exhibiting similar percentage of DNA strand breakage between treatments C and Phe (%DNA in tail > 14%) and

between B[b]F and M (%DNA in tail > 21%) (Figure 4.4 A1). Fish injected with benzo[b]fluoranthene isolated, from experiment A, exhibited a significant increase of percentage of DNA in tail (Fisher's LSD test, $p < 0.05$) compared to the control and Phe treatments (Figure 4.4 A1). Animals exposed to the PAH mixture also showed a significant increment of DNA strand breakage (Fisher's LSD test, $p < 0.05$) when compared to the Phe treatment (Figure 4.4 A1). However, although no significant differences of percentage of tail moment (Fisher's LSD test, $p < 0.05$) were found for fish treated with B[b]F, fish exposed to the PAH mixture showed a significant increase (Fisher's LSD test, $p < 0.05$) compared to the control and Phe treatments (Figure 4.4 A2). Nevertheless, animals injected with benzo[b]fluoranthene and the combination of the two PAHs exhibited a significant increase of percentage of Olive moment (Fisher's LSD test, $p < 0.05$) when compared with the control and Phe treatments (Figure 4.4 A3).

With respect to experiment B, fish injected with phenanthrene revealed an significant increment of percentage of DNA in tail (Fisher's LSD test, $p < 0.05$) compared to the other treatments, however no significant differences were found between control, B[b]F and M treatments (Figure 4.4 B1). Although, animals treated with phenanthrene exhibited a significant increase of percentage of tail moment (Fisher's LSD test, $p < 0.05$) compared to control treatment (Figure 4.4 B2), and a significant increase of percentage of Olive moment (Fisher's LSD test, $p < 0.05$) compared to control and M treatments (Figure 4.4 B3), these tail moments showed no significant differences (Fisher's LSD test, $p < 0.05$) between the B[b]F and Phe treatments, unlike the % DNA in tail (Figure 4.4 B1, B2 and B3). On the other hand, % DNA in tail and Olive tail moment parameters showed significant differences (Fisher's LSD test, $p < 0.05$) between Phe and M treatments, contrary to what was observed for the tail moment parameter (Figure 4.4 B1, B2 and B3).

Overall, in experiment A, B[b]F and M treatments were responsible for the highest induction of DNA strand breakage, as well as, percentage of tail moment and Olive tail moment. Conversely, experiment B induced the highest percentage of DNA in tail, tail moment and Olive tail moment, led by exposure to phenanthrene isolated.

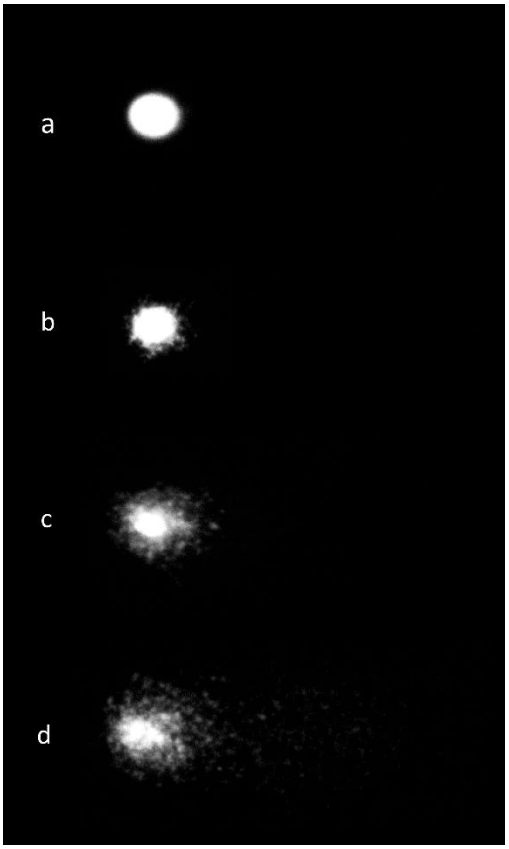

Figure 4.3. Comet examples from injected *Dicentrarchus labrax* showing nucleoids with ≈ 0% (a), ≈ 10% (b), ≈ 25% (c) and ≈ 35% (d) DNA in tail.

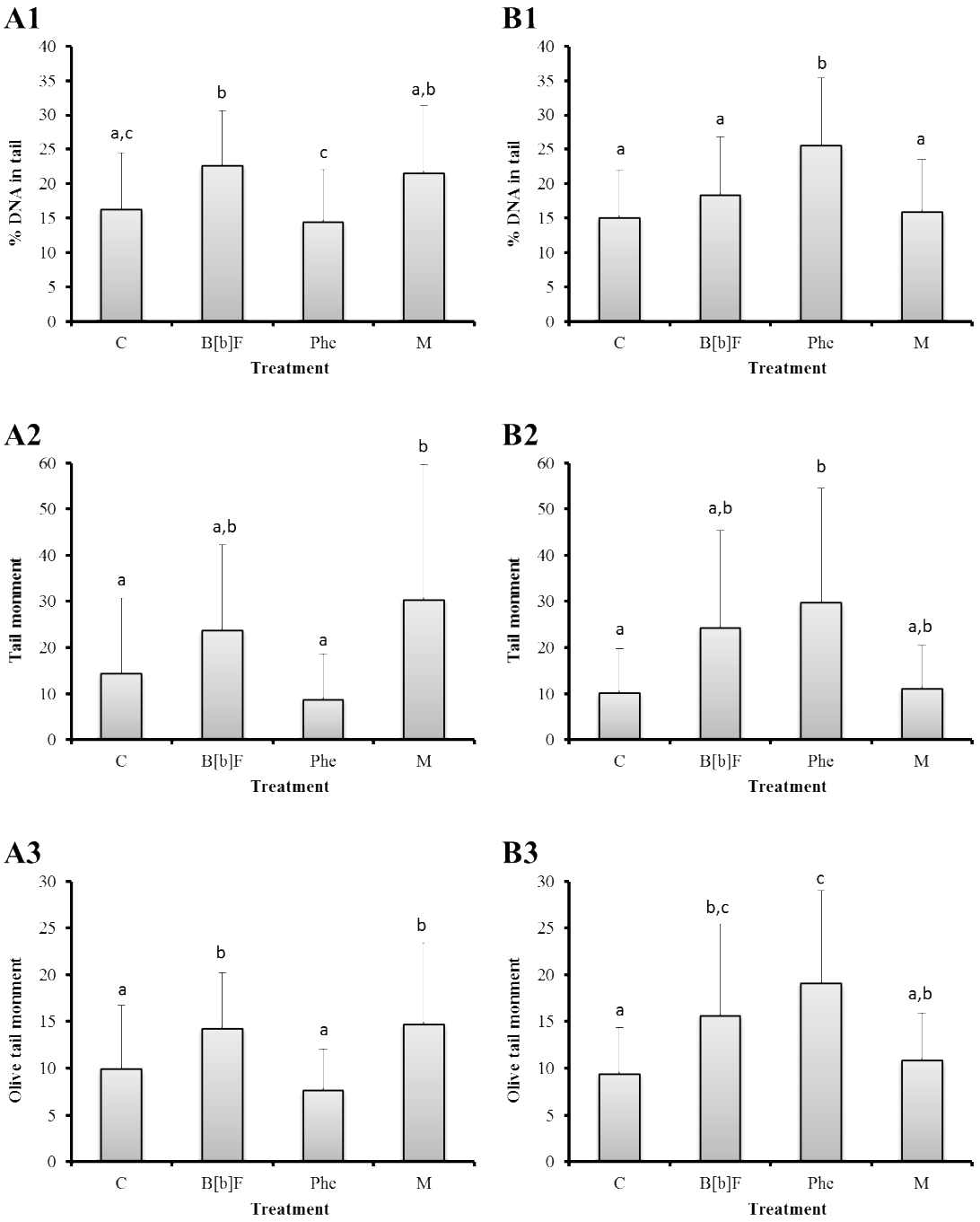

Figure 4.4. Mean percentages of the three Comet assay parameters used (100 comets measured per individual). (A) Experiment with the lower dose (5 $\mu g.g^{-1}$ fish ww). (B) Experiment with the higher dose (10 $\mu g.g^{-1}$ fish ww). (1) Mean percentage of DNA in tail. (2) Mean percentage of tail moment. (3) Mean percentage of olive tail moment. Error bars represent 95% confidence intervals. Different letters indicate significant differences (Fisher's LSD test, $p < 0.05$). C - control treatment. B[b]F - treatment with benzo[b]fluoranthene (higher molecular weight PAH). Phe - treatment with phenanthrene (lower molecular weight PAH). M - treatment with the combination of the two PAH compounds.

4.3. Histopathological alterations

4.3.1. Liver histopathology

Dicentrarchus labrax from the control treatment of either experiment, A and B, presented a normal parenchyma consistent with juveniles, composed of polyhedral hepatocytes with a clear cytoplasm and regular-sized nuclei (Genten *et al.*, 2009). The sea bass liver, similar to that of other fish species, is characterized by the presence of other tissue elements such as, bile ducts and exocrine pancreatic tissue (hepatopancreas) (see Figure 4.5 F). Small capillaries (sinusoids) branching from large blood vessels were observed, containing few blood cells, mostly erythrocytes (Figure 4.5 A). Also, the liver of these animals showed no significant signs of histopathological lesions and alterations. Fish injected with phenanthrene displayed high resemblances to control fish, exhibiting little or no signs of inflammatory response (Figure 4.5 B), indicated by infiltration of leukocytes and, typically, by congested adjacent blood vessels (hyperemia). Likewise, livers of fish treated with phenanthrene from experiment A (low dose) displayed high similarities with fish from experiment B (high dose) of the same treatment. However, fish injected with benzo[b]fluoranthene and the PAH mixture presented greater severity and dissemination of lesions and alterations in the hepatic parenchyma than the control animals. Overall, livers of fish exposed to the PAH mixture from experiment B (high dose) showed more damage-related lesions than fish injected with the PAH mixture from experiment A (low dose).

Between all of alterations most usually observed in livers of fish injected with PAHs, circulatory disturbances and fat vacuolation were the most conspicuous. Although, inflammatory response-related alterations, such as hyperemia and infiltration of defense cells, was observed in all treatments, B[b]F and the PAH mixture treatments presented signs of greater inflammatory response (Figure 4.5 C, D, E and F). For these treatments was frequently observed a more pronounced hyperemia, likely caused by inflammation evidenced by proliferation and swelling of sinusoids. Inflammation occurred in all treatments in both experiments A and B, however limited to small foci in control livers (Figure 4.5 A). Hepatocyte fat vacuolation was observed in livers of all treatments of both experiments A and B, even in some control animals and fish injected with phenanthrene (Figure 4.5 B). However, fish injected with benzo[b]fluoranthene and the PAH mixture presented greater degree and dissemination of this progressive change, exhibiting highly vacuolated hepatocytes, enlarged due to increase lipid storage (Figure 4.5 D and E).

Focal hepatic necrosis was observed in all treatments, but the occurrence and extension of necrotic

areas was more significant in fish exposed to benzo[b]fluoranthene and the PAH mixture, from both experiments A and B (Figure 4.5 C and D). Occasionally, focal hemorrhage was observed in necrotic areas (Figure 4.5 D and F). Nuclear alterations such as pyknosis were recurrent in areas where focal cell death was occurring (necrosis or apoptosis, in some cases), indicating alterations to chromatin (Figure 4.5 C). Livers of fish injected with benzo[b]fluoranthene and the PAH mixture from experiment B (high dose) also presented a higher prevalence of macrophages intruding the damaged tissue, suggesting inflammation (Figure 4.5 D and F). In the most damage livers of fish injected with benzo[b]fluoranthene from experiment B (high dose) was observed different forms of hepatocellular degeneration (Figure 4.5 D). In addition, this particular liver presented lipidic droplets inside blood vessels, which could possibly be an artefact, but nevertheless confirms that was in fact a fat liver (Figure 4.5 D).

One of the fish subjected to the mixture treatment from experiment B (higher dose) presented a small area of a hepatocellular alteration similar to adenomatous tissue (Figure 4.5 E). This focus appeared to be well circumscribed by an area of compressed adjacent parenchyma. However, define diagnosis was impaired by the presence of a branch of the portal triad. In the same specimen, a necrotic focus of pancreatic tissue was also observed, presenting cells with pyknotic nuclei and defense cells infiltrating the damaged tissue (Figure 4.5 F). These two hepatocellular alterations (hepatocellular adenoma and pancreatic necrosis) were only observed in one specimen and, therefore, it is improbable that these liver lesions had been caused by PAH exposure.

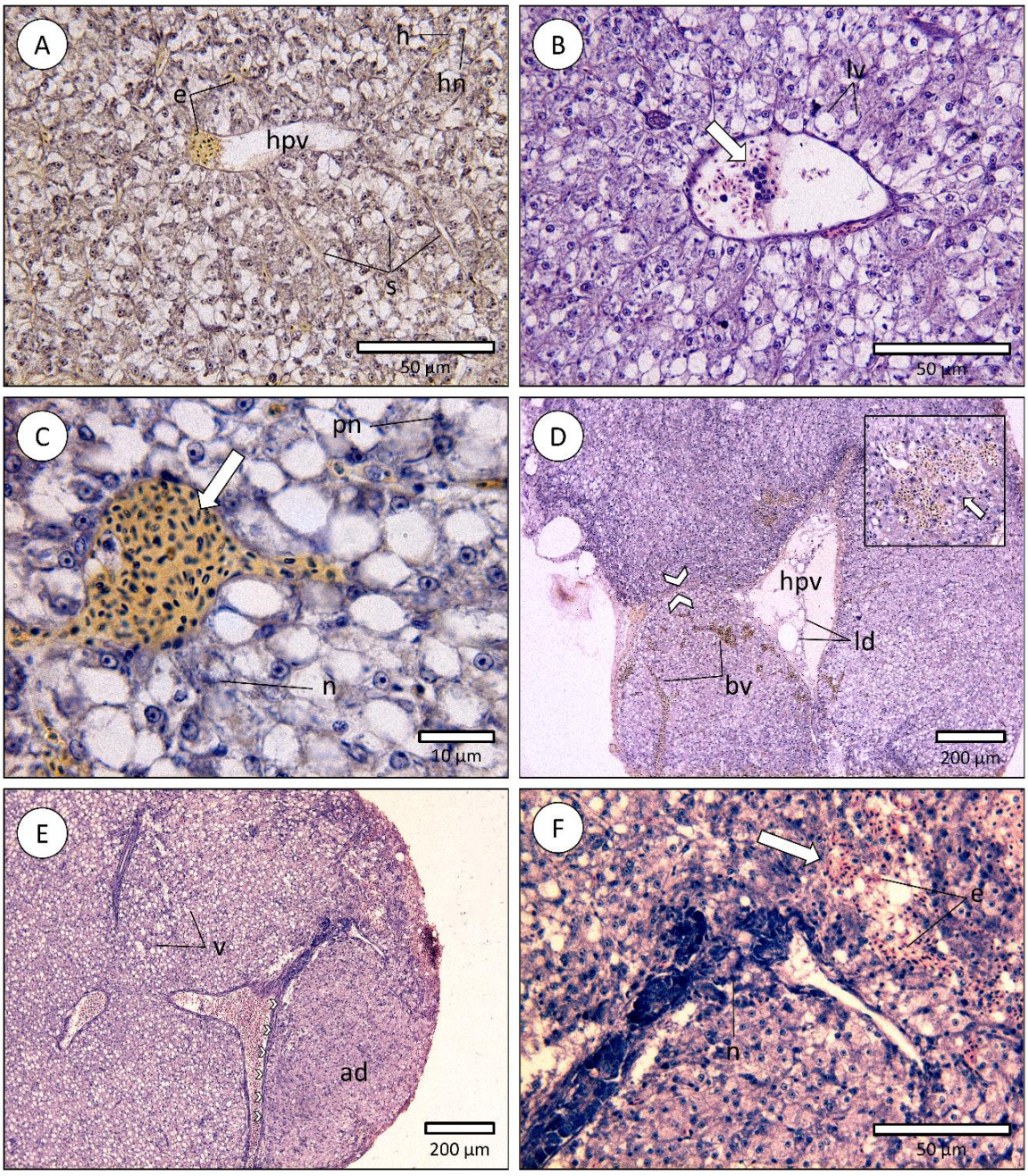

Figure 4.5. Common histopathological lesions and alterations observed in livers of tested *Dicentrarchus labrax* (H&E). (A) Overall aspect of the morphology of a normal juvenile liver (control fishes) from experiment A (low dose), exhibiting well-defined hepatocytes (h), roughly polyedric in shape, bearing a clear cytoplasm and a spherical nucleus (hn). Sinusoids (s) may be seen branching from the hepatic portal vein (hpv) containing a few erythrocytes (e). (B) Liver of a fish injected with phenanthrene from experiment A (low dose) exhibiting a moderate inflammatory response, revealed by a few infiltrating defense cells (arrow). Lipid vacuoles (lv) can also be observed, appearing as large empty structures inside cells. (C) Focal hepatic necrosis (n) from a fish exposed to benzo[b]fluoranthene from experiment A (low dose). Nuclear pleomorphisms such as pyknosis (pn) were commonly observed at necrotic areas. Swollen blood vessels with erythrocytes and defense cells, indicate focal hyperemia (arrow). (D) Liver from a fish injected with benzo[b]fluoranthene from experiment B (high dose) presenting different stages of fat vacuolation. Foci of different forms of hepatocellular degeneration are separated by a clear▶

◀border (arrowheads). Proliferation and swelling of blood vessels (bv) are also evident. Lipid droplets (ld) inside hepatic portal vein (hpv), as a consequence of a fatty liver. Inset: hemorrhage characterized by blood cells and defense cells invading the liver parenchyma at a necrotic focus (arrow). (E) Liver from a fish injected with benzo[b]fluoranthene and phenanthrene (mixture treatment) from experiment B (high dose), exhibiting a small area of hepatocellular alteration similar to adenomatous tissue (ad). The anaplastic tissue seems to compress the edge of the normal parenchyma (arrowheads). Proliferation of hepatocytes containing fat vacuoles (v) was also observed. (F) Necrotic focus (n) of pancreatic tissue in the liver of a fish injected with benzo[b]fluoranthene and phenanthrene (mixture treatment) from experiment B (high dose). Hemorrhage (arrow) is indicated by the infiltration of erythrocytes (e) into necrotic tissue.

4.3.2. Spleen histopathology

Overall, the majority of the fish presented the normal spleen microanatomy common among vertebrates, regardless of treatment. The spleen was composed essencially by two components, white and red pulp without clear boundaries, enclosed by a capsule (Figure 4.6 A). The spleen of control animals from both experiments A and B, exihibited increased volume of red pulp relatively to white pulp, comparatively to other treatments, as expected from a normal organ (Figure 4.6 A and B). In the sea bass, white pulp is poorly developed, composed essentially of lymphoid tissue, interconnected by a system of sinusoids and splenic cords forming the red pulp (Quesada *et al.*, 1990).

The most frequent histopathological alteration was hyperemia. This alteration was observed in all treatments from both experiments A and B, however fish injected with benzo[b]fluoranthene and phenanthrene (mixture treatment) presented signs of greater inflammatory response, indicated by a more pronounced hyperemia and infiltration of defense cells, mostly lymphocytes and macrophages, into blood vessels (Figure 4.6 C).

Aggregates of pigmented macrophages, i.e. melanomacrophage centers (MMC), were frequently observed in all treatments, even in control fishes, scattered through the splenic parenchyma. These centers appear as irregular cell clusters in the stroma of the hematopoietic tissue of the spleen, commonly concentrated around the blood vessels. The present a granular pigmented material, with dark brown-black (melanin-like) or brown-yellow (lipofuscin) deposits. Overall, the main pigment in MMCs was lipofuscin, as common in fish (Agius 1979a,b).

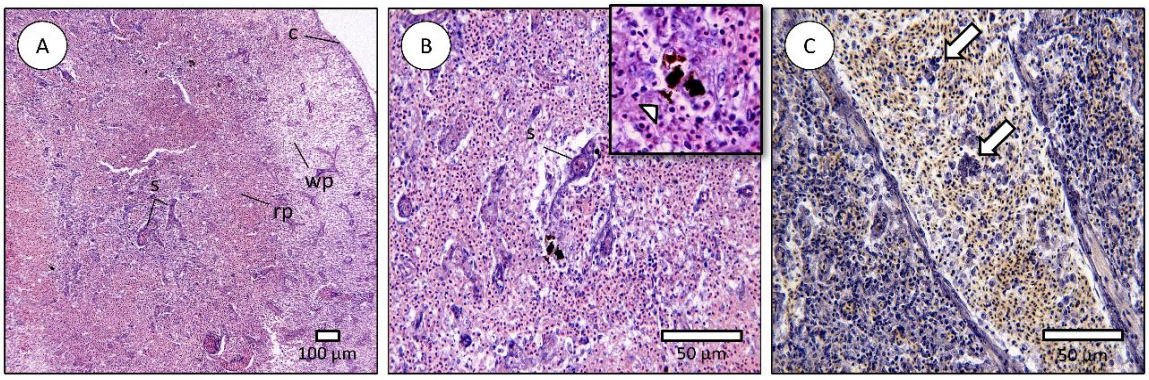

Figure 4.6. Common histopathological lesions and alterations observed in the spleen of tested *Dicentrarchus labrax* (H&E). (A) Overall aspect the normal splenic parenchyma, which is composed by white (wp) and red (rp) pulp, enclosed by a capsule (c). s sinusoids. (B) Hyperemia, here in the spleen of a control fish, which was commonly revealed by congestion of sinusoids (s). Inset: Melanomacrophage centres (arrowhead) were commonly observed scattered through the splenic parenchyma. They are indicated by bodies containing of dark brown (melanin) or brown yellow (lipofuscin) materials. (C) Swollen spleen of a fish injected with benzo[b]fluoranthene and phenanthrene resulting in a high inflammatory response. Agglomerates of inflammatory cells (arrows) were frequently observed in blood vessels of inflammated spleens.

4.4. The Microtox test

The mean acute toxicity of tested compounds, isolated and combined, is presented in Figure 4.7. Both experiments, A and B, presented a similar % effect of toxicity for each treatment (inferred from the loss of *V. fischeri* bioluminescence). Control (DMSO only) and B[b]F treatments had similar acute toxicity with an average of 50% inhibition of luminescence, whereas Phe and M presented 90%. Comparatively, Phe thus caused higher acute toxicity than B[b]F at the tested doses. Still, no clear dose-effect responses were found, i.e. both doses induced similar responses, possible due to the elevated effect baseline caused by DMSO cytotoxicity.

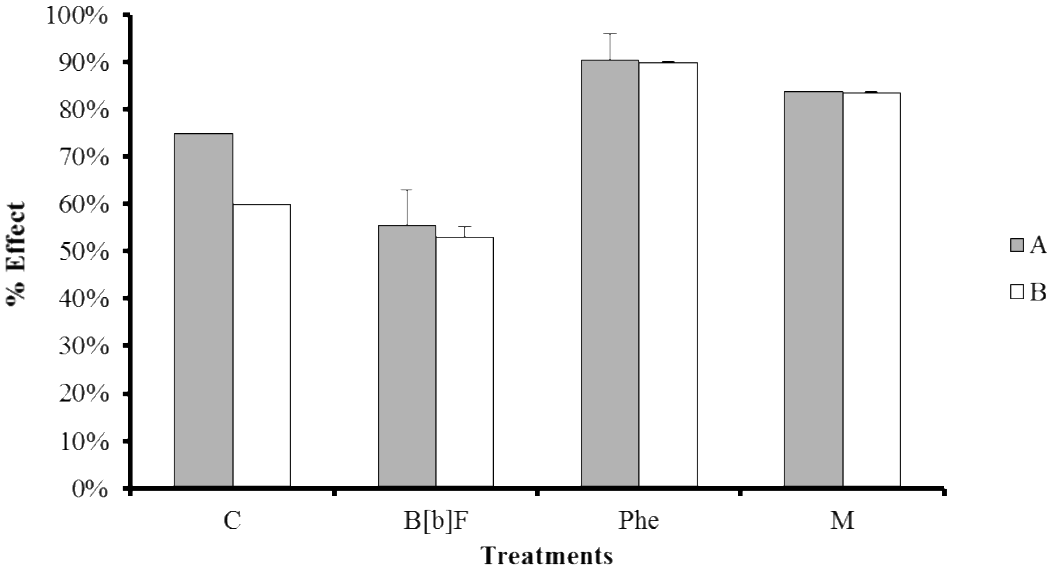

Figure 4.7. Acute toxicity (% inhibition of luminescence of *Vibrio fischeri*) obtained for each treatments C (control treatment), B[b]F (the potentially carcinogenic PAH), Phe (the non-carcinogenic PAH) and M (combination of the two PAH compounds). Results are shown for each experiment: A (low dose, 5 $\mu g.g^{-1}$ fish ww) and B (high dose, 10 $\mu g.g^{-1}$ fish ww). Error bars represent 95% confidence intervals.

5. Discussion

The present study revealed that the two PAHs, benzo[b]fluoranthene (considered carcinogenic) and phenanthrene (non-carcinogenic), isolated or combined, caused different patterns of toxic effects to a marine fish. However, both compounds demonstrated ability to induce DNA damage at the level of nucleotide chains and at the chromosomal level, as well as histopathological alterations. Still, *Dicentrarchus labrax* injected intraperitoneally with benzo[b]fluoranthene only and the combination of benzo[b]fluoranthene and phenanthrene showed, in general, more severe genotoxic and histopathological lesions than fish treated with phenanthrene only. Overall, benzo[b]fluoranthene caused moderate clastogenic and aneugenic alterations and significant hepatic histopathological alterations, especially concerning inflammation-related responses. It must be noted that fish were subjected to a short-time test (48 h) after injection and, therefore, the overall histopathological hepatic alterations are more consistent with acute effects than chronic alterations, which would require longer bioassays to become developed. In spite of the lack of knowledge on the differences between the toxicological pathways of carcinogenic and non-carcinogenic PAHs, the results are accordant with previous findings, under different experimental conditions, on fish (Neuparth *et al*., 2009; Machado *et al*., 2014) and even bivalves (Martins *et al*., 2013), which confirms that both phenanthrene and benzo[b]fluoranthene possess different genotoxic potentials.

The genotoxicity of PAHs is directly dependent of the activity of CYP (cytochrome P450) mixed-function oxygenases (MFOs), which are responsible for the bioactivation of these compounds during phase I of detoxification, producing epoxide intermediate metabolites, that are further converted to highly reactive diol epoxides (Shimada and Fujii-Kuriyama, 2004). In fact, diol epoxides of PAHs, which can affect normal cell division when reacting with DNA to form adducts, have been linked to carcinogenic effects (e.g. Vijayalakshmi and Suresh, 2008). Some studies with mice suggest that some potentially genotoxic phenanthrene metabolites (like dihydrodiols and diol epoxides) less efficiently cross nuclear membranes (resulting in poor interaction with DNA), due to their relatively higher polarity compared to those of larger PAHs such as benzo[b]fluoranthene (considered carcinogenic to humans) (Wood *et al*., 1979). On the other hand, the reactivity of the metabolite (activated PAH) towards the DNA molecule depends on the parent compound and CYP isozymes to which it preferentially binds to. PAH bioactivation can also lead to the production of reactive oxygen species (ROS) as by-products. On their turn ROS may directly or indirectly cause DNA damage via DNA oxidation or metabolic dysregulation, respectively.

It should also be stressed that PAH activation is regulated by a positive feedback loop that regulates CYP transcription. It is known that PAHs may induce CYP1A gene expression through the binding to an intracellular receptor complex, the Aryl Hydrocarbon Receptor (Ahr), which, on its turn, will form a complex with the aryl hydrocarbon nuclear translocator (ARNT). This complex will bind to a specific part of the DNA, the xenobiotic response element (XRE), promoting transcription (see for reviews Goksøyr and Förlin, 1992; Bucheli and Fent, 1995).

Billiard *et al.* (2002) observed that the common model PAH benzo[a]pyrene, also a five-ring PAH like B[b]F, although far more extensively studied, and phenanthrene had differential affinities towards Ahr and, subsequently, different abilities for CYP1A induction in juvenile rainbow trout (*Oncorhynchus mykiss*). Actually, five-ring PAH, like B[a]P and B[b]F, are regarded as stronger Ahr agonists than lower molecular weight PAHs, therefore more able to induce CYP1A transcription and, consequently, the bioactivation of PAHs into genotoxic metabolites (see Mu *et al.*, 2012). This may explain the higher genotoxic damage observed in fish injected with B[b]F when compared to the fish injected with Phe following injection with the lower dose, albeit not the higher dose treatment (Figure 4.4). Nonetheless, it is highly likely that the high-dose treatment induced significant cytotoxic effects in the animals' peripheral blood cells, which is accordant with the results from the Microtox test (Figure 4.7), in spite of the differences between blood cells and the prokaryote *V. fischeri*.

The different affinities that benzo[b]fluoranthene and phenanthrene hold towards Ahr and ability for CYP1A induction in sea bass can also explain why the most severe cases of fat vacuolation where observed only in B[b]F and the PAH mixture treatments from experiment B (high dose) (Figure 4.5 D, E). Since PAHs are highly hydrophobic and tend to accumulate in lipid-rich tissues, such as the liver parenchyma, some researchers suggest that fat vacuolation could be a response mechanism of the organism contaminated to PAHs, to store this liposoluble compounds and/or their metabolites (Köhler, 1990; Biagianti-Risbourg *et al.*, 1995; Costa *et al.*, 2011b). Considering that benzo[b]fluoranthene, being strong Ahr agonists, yielded more metabolites than phenanthrene, and therefore, fish injected with benzo[b]fluoranthene (high molecular weight PAH) and the mixture of PAHs may have suffered from a greater dissemination of fat vacuolation due of exposure to a higher dose, either as an attempt to bioaccumulate the substances or as a consequence of general metabolic failure leading to carbohydrate metabolism dysfunction. In fish, this type of hepatic alteration is a common response to xenobiotic exposure, including PAHs (Marty *et al.*, 2003; Costa *et al.*, 2009; Zorita and Cuevas, 2014).

In fact, Costa *et al.* (2011b) reported that fat vacuolation could be caused by other unspecific factors since fatty livers are a common alteration observed in farmed fish and may depend on diet. In addition, some authors suggest that fatty livers may account for reduced energy production and weakened anti-oxidative responses, potentially leading to hepatocellular dysfunctions (Vendemiale *et al*., 2001; Sánchez-Pérez *et al*., 2005). So, it is possible that control animals already had their fat metabolism altered and as consequence, had presented in their livers some degree of fat vacuolation.

Polycyclic aromatic hydrocarbons are known to induce both DNA strand breakage as well chromosomal breakage, leading to the formation of erythrocytic nuclear abnormalities (ENA) (see, for example, Costa *et al.*, 2008, 2011a; Neuparth *et al.*, 2009). Although, DNA fragmentation, formation of DNA-PAH adducts and chemically altered nucleotide (alkali-labile sites) may result from direct action of mutagens to the DNA chains (e.g. Neuparth *et al.*, 2009; Costa *et al.*, 2011a), chromosomal clastogenesis, assessed through the ENA assay, may be a consequence of the errors occurred during cellular division, where DNA damage was passed on to daughter cells, biomagnifying the initial baseline DNA damage. For this reason, considering that DNA strand breakage damages may repaired depending on type and extent, chromosomal clastogenesis may be considered a more severe type of mutagenesis, since it is unlikely to be repaired (Costa *et al*., 2011a). Therefore, it is likely that benzo[b]fluoranthene can, overall, induce more severe genotoxic damage (Figure 4.2).

Since the frequency of nuclear abnormalities are linked to the frequencies of apoptotic and necrotic cells (e.g. Ghiraldini and Mello, 2010), the greater frequency of ENA observed in blood cells of fish treated with benzo[b]fluoranthene could be linked with acute lesions and alterations of the hepatic parenchyma of fish injected with the same treatment. In fact, focal hepatic necrosis, nuclear pleomorphism and inflammatory response were usually observed together (Figure 4.5 C, D). The presence of nuclear pyknosis and inflammation on livers clearly confirms the evidence of hepatic necrosis and is usually associated with toxicant-induced necrosis (Newman, 2010). It must be noted that necrotic foci allied with nuclear pyknosis and hemorrhage (indicating inflammation) has already been reported in livers of juvenile soles exposed to sediments mostly contaminated by organic compounds (Costa *et al*., 2009, 2011b), indicating that these compounds are strong inducers of severe, likely acute, hepatic lesions albeit unspecific.

Although, benzo[a]pyrene genotoxicity was confirmed in vertebrates (Metcalfe, 1988; Pacheco

and Santos, 1997; Gravato and Santos 2002, 2003) and invertebrates (Venier *et al*., 1997) and PAHs with four or more condensed benzene rings (like B[b]F) are potential mutagenic and/or carcinogenic as benzo[a]pyrene (Gravato and Santos, 2002), present results showed that phenanthrene (PAH with three condensed rings) is also genotoxic to sea bass (by eliciting DNA strand breaks), when fish were injected with higher doses for a short-term period (Figure 4.4), apparently in accordance with the results of the Microtox test (Figure 4.7).

Since phenanthrene is a lower molecular weight PAH, its metabolites are generally acknowledged to be less genotoxic than those of larger PAHs, which results from a poorer interaction with DNA (Thakker *et al*., 1978; Wood *et al*. 1979; Martins *et al*., 2013). It is possible that exposure to benzo[b]fluoranthene yielded both genotoxic metabolites and ROS, while phenanthrene caused genetic damage, mainly, as a result of the formation of ROS alone. Although exposure to PAH might produce reactive oxygen species, under normal physiological conditions they can be removed by antioxidant defense systems (Livingstone, 2001; Meyer *et al*., 2002). Yin *et al*. (2007) observed, in fish (*Carassius auratus*) exposed to different concentrations of phenanthrene, an increase of ROS production, mostly hydroxyl radical ($^{\bullet}$OH). This type of radical is the most reactive towards the DNA molecule (Cadet *et al*., 2010). Yin *et al*. (2007) also demonstrated that fish can experience severe oxidative stress when exposed to high concentrations of phenanthrene. Therefore, it is possible that fish for experiments B, injected with higher doses of phenanthrene, suffered severe oxidative stress, suppressing the antioxidant defense system, and led to a higher induction of total DNA strand breakage (Figure 4.4). Once more, these findings are accordant with the results from the Microtox test (Figure 4.7), indicating higher acute effects induced by this PAH. It should be noted that the high doses administered in sea basses are not ecologically relevant, which, allied with the stress caused by the injection treatments may explain the highest level of mortality in fish treated with phenanthrene and the PAH mixture, from experiment B (Table 4.1).

The exposure of the luminescent marine bacterium *Vibrio fischeri* to phenanthrene appears to pose a higher short-term acute toxicity than benzo[b]fluoranthene. Actually, the biotransformation of phenanthrene, leading to the formation of phenanthrene quinones, leads to inhibition of *V. fischeri* luminescence, even at low concentrations (Wang *et al.*, 2009). Wang *et al*. (2009) demonstrated that phenanthrene quinones posed a greater toxicity to *V. fischeri* than its parent compound, through ROS production. In fact, degradation of phenanthrene by bacteria has been extensively studied (Boldrin *et al*., 1993; Story *et al*., 2001; Kim *et al*., 2003; Kang *et al*., 2003; Seo *et al*., 2009), however information regarding to bacterial biodegradation of PAHs with

five or more rings is still limited. Nevertheless, Seo *et al*. (2009) and Kanaly and Harayama (2000) reported that benzo[a]pyrene, more liposoluble than phenanthrene, poses a higher resistance to microbial degradation, and therefore, the relation between biodegradation rates and PAH molecular weight must be considered in toxicity analyses. For this reason, it is possible that phenanthrene was metabolized by bacterium *V. fischeri* at a higher rate than benzo[b]fluoranthene, yielding more metabolites and present a higher toxic potential to bacteria, as shown by the Microtox test (Figure 4.7).

In contrast, despite the Microtox test revealed a similar acute toxic effect of phenanthrene and the PAH mixture (Figure 4.7), no similarities were found between this pattern of toxicity and those obtained for ENA and the Comet assays in animals from experiment B (high dose) (Figures 4.2 and 4.4). In fact, the present results suggest that interaction effect between the two PAHs depends on the dose of exposure. It should be noticed that, as far other toxicants, PAH interactions effects can be additive (when lead to an addition of the effect of both PAHs), supra-additive, synergistic (when the effect of the two PAHs yields an effect that cannot be attained by either isolates compound) and antagonistic (when a PAH could suppress the effect of the other compound) (Freedman, 1995; Staal *et al.*, 2007; Gonçalves *et al.*, 2008). Even though many studies report additive or synergistic effect in complex PAH mixtures in fish (see e.g. Basu *et al.*, 2001; Billiard *et al*., 2008; Fleming and Di Giulio, 2011), and also in humans (Staal *et al*., 2007), some researchers demonstrated antagonistic interactions of PAHs in multiple organisms, from mice (Springer *et al.*, 1985) to *Salmonella* (Hermann, 1981; White, 2002). From these contradictory reports, it is clear that further research is needed to better understand the behavior of genotoxic compounds in complex mixtures and the relation of dose- and time- effect of PAHs and their interactions.

For animals subject to experiment B, the combined effects of the two PAHs seems to be less genotoxic than the effects of the two isolated compounds, which suggest that interactions between benzo[b]fluoranthene and phenanthrene, in higher doses, can produce antagonistic effects. Staal *et al.* (2007) speculated that when benzo[a]pyrene is combined with other PAHs, can lead to a decrease of B[a]P metabolism and formation of genotoxic metabolites and consequently reduced carcinogenic potential of B[a]P. Therefore, it is possible that the interaction of phenanthrene and benzo[b]fluoranthene, at higher doses, decreased the bioactivation of B[b]F, leading to a reduction in DNA adducts formation (thus, less DNA fragmentation measurable through the Comet assay, at least in part), since less genotoxic metabolites were reduced. This result has been observed, for instance, in human cells, where lower levels of DNA adducts occurred following exposure to

PAH mixtures than cells treated with benzo[a]pyrene alone (Mahadevan *et al.*; 2004).

Actually, some authors suggest that the occurrence of more than one CYP1A inducers can interfere with the integrity of the enzyme causing its inactivation (Stegeman and Hahn, 1994). The inhibition of CYP1A enzymes in fish liver was showed in several studies at high PAH exposure (Haasch *et al.*, 1993; Schlezinger and Stegeman, 2001; Correia *et al.*, 2007). Therefore, it is highly likely that the high-dose treatment of PAH mixture may have led to inhibition of MFOs, generating less active metabolites, hence less DNA strand breakage and chromosomal damage (Figures 4.2 B and 4.4 B). Interestingly, the same was not observed in livers and spleens of fish treated with the mixture of PAH. Overall, fish injected with the PAH mixture, from experiment B, presented a more damage-related lesions than fish treated with lower doses of the two PAHs. The same relation was observed in livers treated with B[b]F only. Overall, livers of fish subjected to experiment B (high dose) presented a greater dissemination of lesions and alterations in the hepatic parenchyma than livers from experiment A (low dose) (Figures 4.5 C, D), suggesting that the damage observed in livers treated with the PAH mixture occurred, mainly, by the exposure to benzo[b]fluoranthene. Since benzo[b]fluoranthene is more lipossoluble (log K_{ow} = 5.78) than phenanthrene (log K_{ow} = 4.52) (IARC, 2010), this PAH tends to accumulate in lipid-rich tissues (as livers) and therefore it is possible that its toxic effect was more evident in liver (yielding acute lesions) than in blood cells (Figures 4.4 and 4.5). Inflammatory response-related alterations, such as hyperemia and infiltration of defense cells, was observed in all treatments, however B[b]F and the PAH mixture presented signs of greater inflammatory response. Although inflammation-related responses and effects are highly non-specific, some researchers already related high prevalence of hyperemia in fish liver as a consequence of exposure to organic compounds (Noreña-Barroso *et al.*, 2004; Agamy, 2012; Zorita and Cuevas, 2014). Even though inflammatory response is usually associated with other pathophysiological processes, in this case, the absence of defense cells other than macrophages indicates that this alteration was probably related to non-infectious agents. It must be noted that even though a probable hepatocellular adenoma (Figure 4.5 E), was found in the liver of a fish treated with the PAH mixture, it is highly unlikely that this hepatic alteration was caused by action of the PAHs, since the animals were allowed to incubate for just 48 h. Still, adenomas have been observed in fish after long-term exposure to PAHs (Reynolds *et al.*, 2003; Zorita and Cuevas, 2010). However this type of hepatocellular change seems to be highly depended of fish age (Vethaak *et al.*, 1996).

Although PAHs are known immunosuppressors to fish (e.g. Carlson *et al.*, 2002a, 2002b, 2004), the present findings indicate reduced alterations to splenic parenchyma, in spite of some moderate

signs of inflammation in fish subjected to the higher doses (Figure 4.6). Still, research on PAH effects to this organ is scarce. It should be noted that not all spleen samples were able to be analyzed by microscope. However, it is probable that PAHs, together with the invasive route of exposure, triggered the immune system of the fish, increasing splenic activity and inflammation.

6. Conclusions

This study aimed at understanding the comparative and interaction effects between two PAHs, isolated or in mixture, in a model organism (*Dicentrarchus labrax*). The present results revealed that isolated benzo[b]fluoranthene (considered potentially carcinogenic to humans) and the PAH mixture caused the most severe genotoxic effects and histopathological alterations in sea bass. However phenanthrene, considered non-carcinogenic to humans by IARC, also poses genotoxic effects, especially at a higher dose (experiment B). The different effects between benzo[b]fluoranthene (five-ring PAH) and phenanthrene (three-ring PAH), with respect to DNA strand breakage and induction of ENAs, confirmed a positive relation between the number of PAH benzene rings and their genotoxicity to fish. Also importantly, the results indicated no significant additive effects between benzo[b]fluoranthene and phenanthrene, under the current experimental conditions, in the contrary, higher doses of PAHs seems to inhibit the activity of CYP1A-related isoenzymes, restraining the formation of genotoxic activated PAH metabolites and consequently, reducing their genotoxicity. The current findings also suggested that the interaction between the two PAHs, in the sea bass, depends on dose of exposure, since the pattern of genotoxic effects varied significantly between the two experimental conditions.

Nevertheless, the results also suggest a distinction between the type of toxicity of the PAHs, in the sense that benzo[b]fluoranthene appears to induce more chronic effects more significantly, whereas phenanthrene is acutely toxic to fish and *Vibrio fischeri*, as shown by higher mortality (especially at higher concentrations) and more significant effects obtained from the Microtox test. However, this issue still requires from further research. Furthermore, *V. fischeri* appears to be highly sensitive to DMSO, in spite of the low concentrations of the carriers used in testing, which may have hindered clear dose-effect relationships.

The Comet assay (alkaline version) seems to be a more sensitive method than ENA analysis, under the present conditions of assessment, since it reflected better the genotoxic effects of benzo[b]fluoranthene and phenanthrene, isolated or in mixture, between the two experiments A and B. The Comet metrics here employed (percentage of DNA in tail, tail moment and Olive tail moment) revealed to be equally good parameters, since they showed a similar pattern of measuring DNA damage at the molecular level, under the current experimental conditions.

The assessment of genotoxicity along with the histopathological effects allowed a better understanding of the global effect of the two PAHs in the sea bass. Both organs, liver and spleen,

showed sensitivity toward PAHs, however liver, likely for being the main organ of accumulation and detoxification of xenobiotics, revealed to be a better target than spleen, since it yielded more severe and diffuse alterations. Still, it must be noted that the lesions and alterations observed are rather unspecific and reflect an acute form of exposure (intraperitoneal injection of the toxicants). Also, peripheral blood cells of fish also proved to be a relevant target in evaluating PAH toxicity, since cell bloods are rapidly affected by the direct action of contaminants and yielded consistent results.

Finally, in the future it is of paramount importance to understand the long-term interaction effects between PAHs under ecologically-relevant scenarios, since PAHs, like many other toxicants, are usually present in the aquatic environment as complex mixture of xenobiotics. Furthermore, prolonged exposures would permit the assessment of fixed chronic lesions such as pre-neoplasms. In accordance, it would be of importance to assess the ability of the animals to recover from chemical challenge, which would involve depuration assays. Altogether, there is still much information lacking on the specific mechanisms underlying PAH interactions, with especial respect to phase I (bioactivation) enzymatic activity and formation of specific metabolites, to which much of the PAH toxicity, genotoxic or not, is owed.

7. References

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